Final Scientific Report

Cover Page

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Project Title:

Elucidating the molecular pathway of sex determination in cultured tilapias and use of genetic markers for creating monosex populations

Investigators	Institutions
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Micha Ron Collaborating Investigator	**A.R.O., Israel** Affiliated Institution

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Abbreviations commonly used in the report, in alphabetical order**:**

Budget: IS: $ **152,000** US: $ **168,000** Total: $ **320,000**

_____ _____

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Final Scientific Report

Abstract (one page maximum, single spaced), include:
List the original objectives, as defined in the approved proposal, and any revisions made at the beginning or during the course of project.

The objectives of this project were to:

1) Identify genetic markers linked to sex-determining genes in various experimental and commercial stocks of *O. niloticus* and *O. aureus*, as well as red tilapias;
2) Develop additional markers tightly linked to these sex determiners, and develop practical, non-destructive genetic tests for identifying genotypic sex in young tilapia;

A third aim, to map sex modifier loci, was removed during budget negotiations at the start of the project.

Background to the topic.
A major obstacle to profitable farming of tilapia is the tendency of females to reproduce at a small size during the production cycle, diverting feed and other resources to a large population of small, unmarketable fish. Several approaches for producing all-male fingerlings have been tried, including interspecific hybridization, hormonal masculinization, and the use of YY-supermale broodstock. Each method has disadvantages that could be overcome with a better understanding of the genetic basis of sex determination in tilapia. The lack of sex-linked markers has been a major impediment in research and development of efficient monosex populations for tilapia culture.

Major conclusions, solutions, achievements.
We identified DNA markers linked to sex determining genes in six closely related species of tilapiine fishes. The mode of sex determination differed among species. In *Oreochromis karongae* and *Tilapia mariae* the sex-determining locus is on linkage group (LG) 3 and the female is heterogametic (WZ-ZZ system). In *O. niloticus* and *T. zillii* the sex-determining locus is on LG1 and the male is heterogametic (XX-XY system). We have nearly identified the series of BAC clones that completely span the region. A more complex pattern was observed in *O. aureus* and *O. mossambicus*, in which markers on both LG1 and LG3 were associated with sex. We found evidence for sex-linked lethal effects on LG1, as well as interactions between loci in the two linkage groups. Comparison of genetic and physical maps demonstrated a broad region of recombination suppression harboring the sex-determining locus on LG3. We also mapped 29 genes that are considered putative regulators of sex determination. *Amh* and *Dmrta2* mapped to separate QTL for sex determination on LG23. The other 27 genes mapped to various linkage groups, but none of them mapped to QTL for sex determination, so they were excluded as candidates for sex determination in these tilapia species.

Implications, both scientific and agricultural.
Phylogenetic analysis suggests that at least two transitions in the mode of sex determination have occurred in the evolution of tilapia species. This variation makes tilapias an excellent model system for studying the evolution of sex chromosomes in vertebrates. The genetic markers we have identified on LG1 in *O. niloticus* accurately diagnose the phenotypic sex and are being used to develop monosex populations of tilapia, and eliminate the tedious steps of progeny testing to verify the genetic sex of broodstock animals.

Achievements

Sex determination in various tilapia strains

We analyzed progeny from 14 families of eight strains from six different species of tilapia (Table 1). Each fish was sexed and then genotyped for microsatellite DNA markers selected from the published linkage map of tilapia (Lee et al., 2005). By examining the inheritance of parental alleles in each family we determined whether the mode of sex determination in each strain followed a male or female heterogametic mechanism. Strong associations were found between sex and the segregation of particular microsatellite markers in each species, indicating a strong genetic basis for sex determination. However, there is variation among strains and species in whether the sex determining loci are found on LG1, LG3 or both linkage groups. Variation was found also in the mode of inheritance, with heterogametic males (XX-XY), heterogametic females (WZ-ZZ) and more complex systems (Table 1).

In *T. mariae*, *O. karongae*, and the Israeli strain of *O. aureus*, sex-linked markers on LG3 predicted the sex of ~95% of the offspring. These species have a maternal heterogametic sex determination mechanism (WZ-ZZ). In the two strains of *O. niloticus*, and in *Tilapia zillii*, sex-linked markers on LG1 predicted the sex of ~90% of the offspring. These species have a paternal heterogametic sex determination mechanism (XX-XY). However, our previous work suggests that this locus will not explain sex determination in all families of the Egyptian strain of *O. niloticus* (Lee et al., 2003).

We previously reported a family of *O. aureus* in which there was an epistatic interaction between the WZ system on LG3 and an XY system on LG1 (Lee et al., 2004). In the current study, we found that markers on LG1 explained the sex of most individuals in *O. mossambicus* and the Egyptian strain of *O. aureus*, but weak associations were found also between sex and markers on LG3. In these two species, the several markers on LG1 showed unusual patterns of inheritance, and further analysis showed strongly deleterious haplotype combinations on LG1.

In order to confirm that LG1 and LG3 are two different, unlinked chromosomes, we physically mapped the sex-linked markers by fluorescent *in situ* hybridization (FISH). The tilapia BAC libraries were screened using the sex-linked markers, and individual BACs were hybridized in pairs to tilapia metaphase spreads. These experiments demonstrated that LG3 corresponds to the largest chromosome in the tilapia karyotype, and that LG1 corresponds to a

different, smaller chromosome. We suggest that the incomplete pairing of the large chromosome bivalent observed by others may reflect a prior evolutionary history as a differentiated sex chromosome, and that a new sex determiner has recently evolved on a smaller chromosome in these species. Although the WZ homologues of LG3 in *O. aureus* remain cytologically indistinguishable, pair-wise hybridization of the sex-linked markers in LG3 revealed a large region of recombination suppression. Markers in a 17 cM region encompassing the sex-determining locus hybridized so specific locations across most of the long chromosomal arm. Markers on the remaining ~80 cM of the linkage map hybridized to a small region encompassing just 25% of the chromosome. We also found sex-specific differences in recombination rate in *O. aureus* (Israeli strain), with a lower rate of recombination in the female (the heterogametic sex in this species). In contrast, we saw no evidence of recombination suppression on the XY chromosomes of *O. niloticus* (Cnaani et al., submitted).

Table 1. The sex determination system and the linkage group location of the sex determination region in eight tilapia strains that were analyzed in this study.

Species (country of origin)	LG1	LG3	Number of Families
O. aureus (Israel)	*	WZ-ZZ	4
O. aureus (Egypt)	**	**	2
O. karongae (Lake Malawi)	N/A	WZ-ZZ	1
O. mossambicus (South Africa)	**	**	3
O. niloticus (Egypt)	XX-XY	N/A	4
O. niloticus (Ghana)	XX-XY	N/A	1
T. mariae (West Africa)	N/A	WZ-ZZ	1
T. zillii (Egypt)	XX-XY	N/A	1

* Markers on LG1 were associated with sex (XX-XY system) only in one out of four tested families.
** Markers on both linkage groups were associated with sex. A lethal effect was observed on LG1 and the heterogametic sex could not be determined.
N/A - no association.

Candidate genes for sex determination

Recent studies revealed that the major genes of the mammalian sex determination pathway are also involved in sex determination of fish. In order to expand our search for the sex determining genes in tilapia, we selected 29 candidate genes for mapping and genotyped these sites for 90 individuals of an F_2 mapping family. The mapping of *Dax1* merged LG16 and LG21 into a single linkage group. The *Amh* and *Dmrta2* genes were mapped to LG23, adjacent to each of two previously reported QTL regions for sex determination (Shirak et al., 2006). The other 27 genes mapped to various linkage groups, but none of them mapped to known QTL for sex determination, so they were excluded as candidates for sex determination in these tilapia species (Cnaani et al., 2007; Shirak et al., unpublished).

Cloning the XY locus on LG1

The major sex-determining region was mapped within an 11 cM interval on LG1 between markers GM201 and UNH995 (Lee et al., 2003). We used a comparative mapping approach to develop new markers in this interval. We screened tilapia BAC libraries with marker UNH995 and recovered a reliable contig of 22 BAC clones. Shotgun sequencing of these clones identified synteny with a region of *Tetraodon* chr5 from 2.7 Mb to 4.8 Mbp. We then identified cichlid ESTs which mapped to this region, and developed SNPs in eight genes. These markers allowed us to further narrow the sex-determining region to 2.6 cM interval between markers BJ702072 and BJ675743. This region represents about 400kb in the *Tetraodon* genome, and corresponds to about 1 Mbp in the tilapia genome. We have four informative EST markers within this region, but have not yet identified any recombinants which narrow the interval. We also used these ESTs as probes to develop a physical map across the region. BAC clones containing these markers assembled into 3 separate contigs which could be oriented on the *Tetraodon* sequence by determining the marker content of individual BAC clones (Lee and Kocher, 2007b). The three remaining gaps in the physical map are being closed by chromosome walking in the BAC libraries. Two candidate genes mapped to this QTL - *Wilms tumor* (*WT1b*) and *ovarian cytochrome P450 aromatase* (*CYP19A1*) were excluded as candidates for sex determining genes due to recombination.
The genetic markers we have identified on LG1 in *O. niloticus* accurately diagnose the phenotypic sex and are being used to develop monosex populations of tilapia, and eliminate the tedious steps of progeny testing to verify the genetic sex of broodstock animals.

Description of Cooperation

Project tasks are carried out in a complementary manner by both teams. Cooperation takes the form of exchange of biological materials (fish, tissue samples, extracted DNA, primers); exchange of data and information, consultations and discussions through e-mail correspondence, phone calls, meetings of team members; and sharing bioinformatics infrastructures developed at UNH. The BARD-sponsored workshop on: "Aquaculture genetics – status and prospects", held in Israel in February 2006, enabled a team meeting attended by the three investigators (Hulata, Kocher, Ron) and two scientists working in the project (Cnaani, Shirak).

References

Lee BY, Penman DJ, Kocher TD (2003). Identification of a sex-determining region in Nile tilapia (*Oreochromis niloticus*) using bulked segregant analysis. Animal Genetics 34: 379-383.

Lee BY, Hulata G, Kocher TD (2004). Two unlinked loci controlling the sex of blue tilapia (*Oreochromis aureus*). Heredity 92: 543-549.

Lee BY, Lee WJ, Streelman JT, Carleton KL, Howe AE, Hulata G, Slettan A, Terai Y, Kocher TD (2005). A second-generation genetic linkage map of tilapia (*Oreochromis* spp.). Genetics 170: 237-244.

Lee BY, Kocher TD (2007a). Exclusion of *Wilms tumor* (*WT1b*) and *ovarian cytochrome P450 aromatase* (*CYP19A1*) as candidates for sex determining genes in Nile tilapia (*Oreochromis niloticus*). *Animal Genetics,* **38**: 85-86.

Lee BY, Kocher TD (2007b). Comparative genomics and positional cloning, pp. 325-337, In: J. Liu (Ed.) *Aquaculture Genome Technologies*. Blackwell.

Shirak A, Seroussi E, Cnaani A, Howe AE, Domokhovsky R, Zilberman N, Kocher TD, Hulata G, Ron M (2006). *Amh* and *Dmrta2* genes map to tilapia (*Oreochromis* spp.) linkage group 23 within QTL regions for sex determination. Genetics 174: 1573-1581.

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	2	1	1	4
Submitted, in review, in preparation	1			1
Invited review papers				
Book chapters		1		1
Books				
Master theses				
Ph.D. theses				
Abstracts	2	2		4
Not refereed (proceedings, reports, etc.)		1		1

Publication details

Papers

Cnaani, A., B.-Y. Lee, C. Ozouf-Costaz, C. Bonillo, J.F. Baroiller, H. D'Cotta, and T.D. Kocher (2007). Mapping of *sox2* and *sox14* in tilapia (*Oreochromis* spp.). *Sex. Develop.*, **1**: 207-210.

Cnaani, A., B.-Y. Lee, N. Zilberman, C. Ozouf-Costaz, G. Hulata, M. Ron, A. D'Hont, J.-F. Baroiller, H. D'Cotta, D.J. Penman, E. Tomasino, J.-P. Coutanceau, E. Pepey, A. Shirak, and T.D. Kocher (2007). Rapid evolution of the sex determination mechanism among closely related cichlid fish species. *Sex. Develop.*, **submitted**.

Lee, B.-Y., and T.D. Kocher (2007a). Exclusion of *Wilms tumor* (*WT1b*) and *ovarian cytochrome P450 aromatase* (*CYP19A1*) as candidates for sex determining genes in Nile tilapia (*Oreochromis niloticus*). *Animal Genetics,* **38**: 85-86.

Lee, B.-Y., and T.D. Kocher (2007b). Comparative genomics and positional cloning, pp. 325-337, In: J. Liu (Ed.) *Aquaculture Genome Technologies*. Blackwell.

Shirak , A., E. Seroussi , A. Cnaani , A.E. Howe, R. Domokhovsky, N. Zilberman , T.D. Kocher, G. Hulata, and M. Ron (2006). *Amh* and *Dmrta2* genes map to tilapia (*Oreochromis* spp.) linkage group 23 within QTL regions for sex determination. *Genetics*, **174**: 1573-1581.

Zilberman, N., S. Reikhav, G. Hulata, and M. Ron (2006). High-throughput genomic DNA extraction protocol from tilapias fin tissue (Technical paper). *Aquaculture,* **255**: 597-599.

Presentations

Cnaani, A., B.-Y. Lee, N. Zilberman, J.-P. Coutanceau, C. Ozouf-Costaz, A. Shirak, G. Hulata, M. Ron, and T.D. Kocher (2006). The genetic basis of sex determination in different tilapia species. *PAGXIV, San Diego, CA, USA.*

Kocher, T.D., B.-Y. Lee, and A. Cnaani (2006). Genomic approaches to identifying sex-determination genes in tilapia. *BARD Workshop "Genetics in Aquaculture: Status and prospects", Eilat, Israel, February 2006*

Lee, B.-Y., and T.D. Kocher (2006). Comparative mapping of sex-determining region in Nile tilapia (*Oreochromis niloticus*). *PAGXIV, San Diego, CA, USA.*

Shirak , A., E. Seroussi , N. Zilberman , G. Hulata, M. Ron , A. Cnaani , A.E. Howe, and T.D. Kocher (2006). Searching for master key regulator genes in the sex determination pathway in tilapias. *BARD Workshop "Genetics in Aquaculture: Status and prospects", Eilat, Israel, February 2006*

Shirak , A., E. Seroussi , N. Zilberman , R. Domokhovsky, A. Cnaani , T.D. Kocher, M. Ron , and G. Hulata (2007). Mapping of candidate genes for sex determination in tilapias. *9th International Symposium on Genetics in Aquaculture. Montpellier, France, June 2006.*

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Avner Cnaani

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Description of Cooperation:

Project tasks were carried out in a complementary manner by both teams. Cooperation took the form of exchange of biological materials (fish, tissue samples, extracted DNA, primers); exchange of data and information, consultations and discussions through e-mail correspondence, phone calls, meetings of team members; sharing bioinformatics infrastructures developed at UNH. The BARD-sponsored workshop on 'Aquaculture genetics – status and prospects' held in Israel in February 2006 enabled a team meeting attended by the three investigators (Hulata, Kocher, Ron) and two scientists working in the project (Cnaani, Shirak).

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Appendix (technical information supporting the research findings): Provide a table of contents and include the following:
Published papers.
One copy of each 'in press', 'accepted' or 'submitted' paper.
Unpublished data, briefly summarized.
Other relevant material may be included (1.5 space, font not smaller than 12).

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